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Advancing forensic accuracy: mitigating methemoglobin interference in postmortem carbon monoxide analysis using sodium dithionite reduction

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Analyzing carbon monoxide concentration within an individual is crucial. The analysis of CO content in a tissue sample is performed using gas chromatography. The concentration is calculated based on a linear equation derived from the calibration curve created with the CO-fortified sample. However, when methemoglobin (MetHb) is formed from putrefaction, it inhibits CO binding to the sample and may lead to inaccurate results. MetHb results from the iron oxidation of normal heme hemoglobin (HHb), and by treating the sample with a reducing agent, it can be converted back to HHb. To investigate the effect of the reducing agent on spleen CO analysis, each sample was divided into two parts. One was treated with a 0.574 M sodium dithionite solution ($\text{Na}_2\text{S}_2\text{O}_4$), a reduced sample, and the other was treated with a rinse solution, serving as the control sample, with both undergoing the same preparation process and analyzed using Gas Chromatography with a Thermal Conductivity Detector (GC-TCD). Spleen samples from 60 autopsy cases were analyzed. The results indicated that 48 cases showed lower CO levels when the sample was reduced compared to the control sample, where the difference of the control and reduced samples ranged from 2.21 to 93.24%, with a median value of 13.83%. 12 cases exhibited no difference, where the difference between control and reduced sample ranged from 0.05 to 1.57%, with a median value of 0.67%. Our findings demonstrate that MetHb formed during decomposition can significantly inhibit CO binding in spleen tissue, leading to overestimation of CO levels when no reducing agent is used. Therefore, incorporating sodium dithionite treatment into GC-TCD methods improves the accuracy of postmortem CO quantification, particularly in putrefied samples.

Keywords Carbon monoxide, Carboxyhemoglobin, Postmortem, Spleen, Reducing agent, GC

Carbon monoxide (CO) is an odorless gas produced by incomplete combustion of hydrocarbons¹. When inhaled, it binds to ferrous ions (Fe^{2+}) in hemoglobin to form carboxyhemoglobin (COHb) and inhibits the formation of oxyhemoglobin (O_2Hb), which is what transfers oxygen through the bloodstream². The main organs affected by hypoxia are the brain and heart due to their oxygen-demanding nature, which explains the lethality of CO and its name, the “silent killer”³. Whether carbon monoxide poisoning was a critical cause of death is determined by analyzing its concentration within an individual’s system. Although oximeters are occasionally used in some jurisdictions for postmortem CO analysis, their accuracy is highly dependent on the quality of the blood sample. In many forensic cases, particularly when decomposition or hemolysis has occurred, the condition of the blood often precludes their use^{4,5}. In this case, the CO content of the tissue sample is analyzed using gas chromatography (GC) with various detectors, including thermal conductivity detector (TCD), mass spectrometry (MS), and flame ionization detector (FID)^{6–10}. It is important to note that a standard Flame Ionization Detector (FID) is not suitable for detecting CO due to its inability to respond to inorganic gases. Only modified FIDs coupled with a methanizer (O-FID) are capable of detecting CO, which limits their widespread use in forensic laboratories.

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compared to TCD-based systems. The spleens were selected as the most optimum organ for tissue samples because of their abundance in red blood cells¹¹.

In the process of analyzing CO with GC, a calibration curve is constructed using the tissue sample fortified with pure CO gas. Fortification refers to the controlled introduction of pure CO gas into biological samples, such as blood or tissue, to enable binding of CO to hemoglobin. However, the success of CO fortification is highly dependent on the condition of the sample. In particular, the oxidation of hemoglobin into methemoglobin during sample degradation can significantly impair CO binding affinity. This oxidative alteration reduces the number of functional heme sites available for CO attachment, resulting in suboptimal CO uptake during fortification. Consequently, the prepared calibration samples may contain lower-than-expected CO levels, thereby affecting the accuracy and reliability of the resulting calibration curve. This can cause the calculated value to be inaccurate, which may result in falsely determining the sample as CO-positive or the area of the sample's chromatogram exceeding the calibration point of 100%.

Typical blood consists of four main types of hemoglobin. Heme hemoglobin (HHb), a normal hemoglobin ready to bind with oxygen or CO; carboxyhemoglobin (COHb), a CO-bound hemoglobin; oxyhemoglobin (O₂Hb), an oxygen-bound hemoglobin, and methemoglobin (MetHb), an oxidized hemoglobin. MetHb is a type of hemoglobin where the ferrous ion (Fe²⁺) in the heme is oxidized to ferric ion (Fe³⁺) and makes it impossible for oxygen or CO to bind, resulting in death or, in this case, unsuccessful fortification^{12,13}. MetHb needs to be lowered to analyze CO accurately, which can be done by treating the sample with a reducing agent to complement the oxidation^{14,15}. Converting MetHb to normal hemoglobin will restore its CO-binding ability and make the fortification more successful. An accurate calibration curve will be constructed, the unusually high calculated CO will be lowered, and the results over 100% and false positives will be fixed.

Before making any changes to the current method, a review of the preceding literature was done regarding CO analysis using GC, and the ones with notable features were selected to be put in Table 1^{16–24}. Though varied in specifics, all methods included a fortification process and a liberating agent, a solution added to release the CO from the sample to the gas phase of the vial. Some used hemolytic agents to completely break down the red blood cells, and some used reducing agents to lower MetHb. A previous study emphasizes the importance of incorporating a reducing agent by describing the limitations of high MetHb formed by postmortem putrefaction¹⁷. The study compares the results of GC and UV, showing that the sample with higher MetHb had a more significant difference between the two analysis results. In addition, it shows that the sample treated with sodium dithionite solution, a reducing agent, had similar results in GC and UV. Therefore, this showcases that the error caused by high MetHb in CO analysis using GC can be compensated by incorporating a reducing agent.

This research was conducted to test the suitability of applying a reducing agent to the current methods used in the National Forensic Service. Based on a literature review, sodium dithionite was selected as a reducing agent. A thermal conductivity detector for GC analysis was used because of its good repeatability²⁵. A preliminary test was conducted on blood to determine the optimal concentration of sodium dithionite (Na₂S₂O₄), and this determined concentration was subsequently tested on 60 spleen samples to compare the results with and without the reducing agent. While GC-TCD methods using sodium dithionite have been described in the literature, these have been largely limited to blood or controlled laboratory samples. In contrast, our study demonstrates the practical applicability of spleen tissue as a reliable matrix in routine forensic science, especially in decomposed bodies, through the analysis of 60 real-world cases.

Reference	Hemolytic	Reducing	Liberating	Others	Saturation	Time(min)	Detector
Collison (1968)	Sterox		K ₃ Fe(CN) ₆	H ₃ BO ₃ , NaCl	Syringe	45	GC-FID
Blackmore (1970)	Saponin, Triton x-100		NaHCO ₃ , Na ₂ CO ₃		CO bubble		GC-TCD
Wu (1992)			K ₃ Fe(CN) ₆	Zeroing solution	Tonometer	35	GC-FID
Middleberg (1993)			H ₂ SO ₄		Inject	55	GC-MS
Sundin AM (2002)			H ₂ SO ₄	tri-Sodium citrate dihydrate	Inject	40	GC-FID
Lewis (2004)	Saponin	Na ₂ S ₂ O ₄	H ₂ SO ₄		CO purge	100	GC-TCD
Vreman (2006)			K ₃ Fe(CN) ₆ , C ₇ H ₆ O ₆ S		Tonometer		GC-FID, RGA
Walch (2010)	Saponin		H ₂ SO ₄		Inject	30	GC-FID
Oliverio (2018)			H ₂ SO ₄		CO bubble	90	GC-MS
Zanaboni (2020)		Na ₂ S ₂ O ₄	H ₂ SO ₄	H ₃ BO ₃	CO bubble	12	GC-TCD

Table 1. Selected studies of CO analysis methods using GC and the chemicals used.

Materials and methods

Reagents and supplies

The chemicals used were sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$; Fischer Scientific Co., USA) and potassium ferricyanide III ($\text{K}_3[\text{Fe}(\text{CN})_6]$; Sigma-Aldrich, Germany). Rinse solution from RADIOMETER(Denmark) was used for sample dilution, and distilled water was used to prepare all reagent solutions. The reducing agent was 0.574 M sodium dithionite solution, and the liberating agent was 10% (w/v) ferricyanide solution. Both solutions were freshly prepared each day at the start of the experiment. Tedlar bags (SUPELCO, USA) were used to contain pure nitrogen and carbon monoxide gas. Henke 60 mL syringe (Henke-Sass, Wolf GmbH, Germany) was used to contain the sample for fortification and a 3-way stopcock from Double Safe, Kaju Healthcare CO., Ltd. (South Korea) was used to close the syringe airtight when dealing with gas. For fortification, a rotator from FINEPCR (South Korea) was used at 40 rpm.

Preparation of spleen samples

The spleen samples were collected from the cadavers subjected to autopsy at the Forensic Medicine Unit of the Daejeon District Office, National Forensic Service. Approximately 10 g fragments of spleen were collected during autopsy, placed directly into labeled sterile polypropylene (plastic) airtight containers, immediately sealed, and stored at 4°C under controlled conditions until the time of analysis. The samples were stored without homogenization, chemical treatment, additives, or stabilizers to avoid potential interference with CO quantification. All samples were processed just prior to analysis, and their use was conducted in accordance with the policies and procedures approved by the Institutional Review Board of the National Forensic Service (IRB NO 906-240131-BR-010-01). The experimental methods were modified from a previously published protocol to include reducing agents during sample preparation, aiming to assess their effect on CO quantification and forensic applicability in decomposed spleen tissue²⁸. Each spleen sample was diced using surgical scissors and squeeze-filtered through sterilized gauze to produce liquid spleen samples. The liquidized sample was halved, and each was put in a 60 mL syringe. The one marked as “reduced” was treated with 100 μL of reducing agent per 1 mL of the sample. The one marked as “control” had 100 μL of rinse solution added per 1 mL of the sample. Both were then vortexed to mix thoroughly. 1 mL of the mixed sample was collected from each syringe and placed in a 10 mL glass vial containing 1 mL of rinse solution to make the test vial.

Preparation of calibration samples

Syringes containing the samples were closed airtight by placing a 3-way stopcock before the needle. The stopcock valve was opened to remove the air and fill the syringe with CO gas from a tedlar bag. The process was repeated twice to put in as much CO as possible, and then the stopcock was closed. For fortification, the syringes were put on a rotator at 40 rpm for 20 min. Subsequently, the same process was done using nitrogen gas but rotated for 10 min to purge the sample and remove the residual CO that didn't bind with hemoglobin. Fortified samples in the syringes were put in headspace vials. Five calibration samples of 10, 30, 50, 70, and 100% were put together by making aliquots of the fortified sample of 100, 300, 500, 700, and 1000 μL and filling up with rinse solution to make a total volume of 2 mL in each vial (Fig. 1).

Instrumental analysis

To analyze CO using GC, the liberating agent is added to free the bound CO to the gas phase of the vial from the COHb of the sample. 1 mL of potassium ferricyanide solution 10% (w/v) was injected into each vial of test and calibration samples. The vials were sealed immediately to prevent the leak. The CO concentration was calculated using the standard calibration curve equation constructed based on the integrated area of the chromatogram of the calibration vials. The instrumental analysis was conducted under the conditions summarized in Table 2.

Method validation

To evaluate the reliability of the analytical method, method validation was performed for spleen (two independent samples) and blood. Key validation parameters, including the limit of detection (LOD), limit of quantification (LOQ), precision (RSD), linearity(R^2), and recovery, are summarized in Table 3. The method exhibited excellent linearity across all matrices ($R^2 > 0.995$), with acceptable precision ($\text{RSD} < 3\%$) and satisfactory recovery ranging from 94.91 to 102.7%. These results demonstrate that the GC-TCD method applied in this study is suitable for the quantification of CO.

Results

Whether the reducing agent played any role in the sample was determined by comparing the results of 60 spleen samples. Each liquidized sample was divided in half to treat one with a reducing agent, a reduced sample, and the other with a rinse solution, a control sample. The identical process of CO fortification was done, and no extra liberation time was spent other than the sample equilibration in the GC cycle. CO eluted at approximately 6 min, but in case other gases were detected, the total run time was 16 min.

48 out of 60 cases had a decrease in the reduced samples, and 12 did not show any changes. Results with a difference more prominent than 2% were grouped as “shifted,” and the differences more minor than that were grouped as “not shifted.”

In the 12 not-shifted cases, the difference was 0.05–1.57%, and the median value was 0.67%. The institute's current CO analysis considers a difference of less than 2% to be the same result. It can be assumed that in these 12 cases, the reduction did not affect the outcome because the MetHb was not high in the first place.

In the 48 shifted cases, the difference was 2.21–93.24%, and the median value was 13.83%. 15 cases had the CO concentration of the control sample higher than 50%, where the average difference between the control and

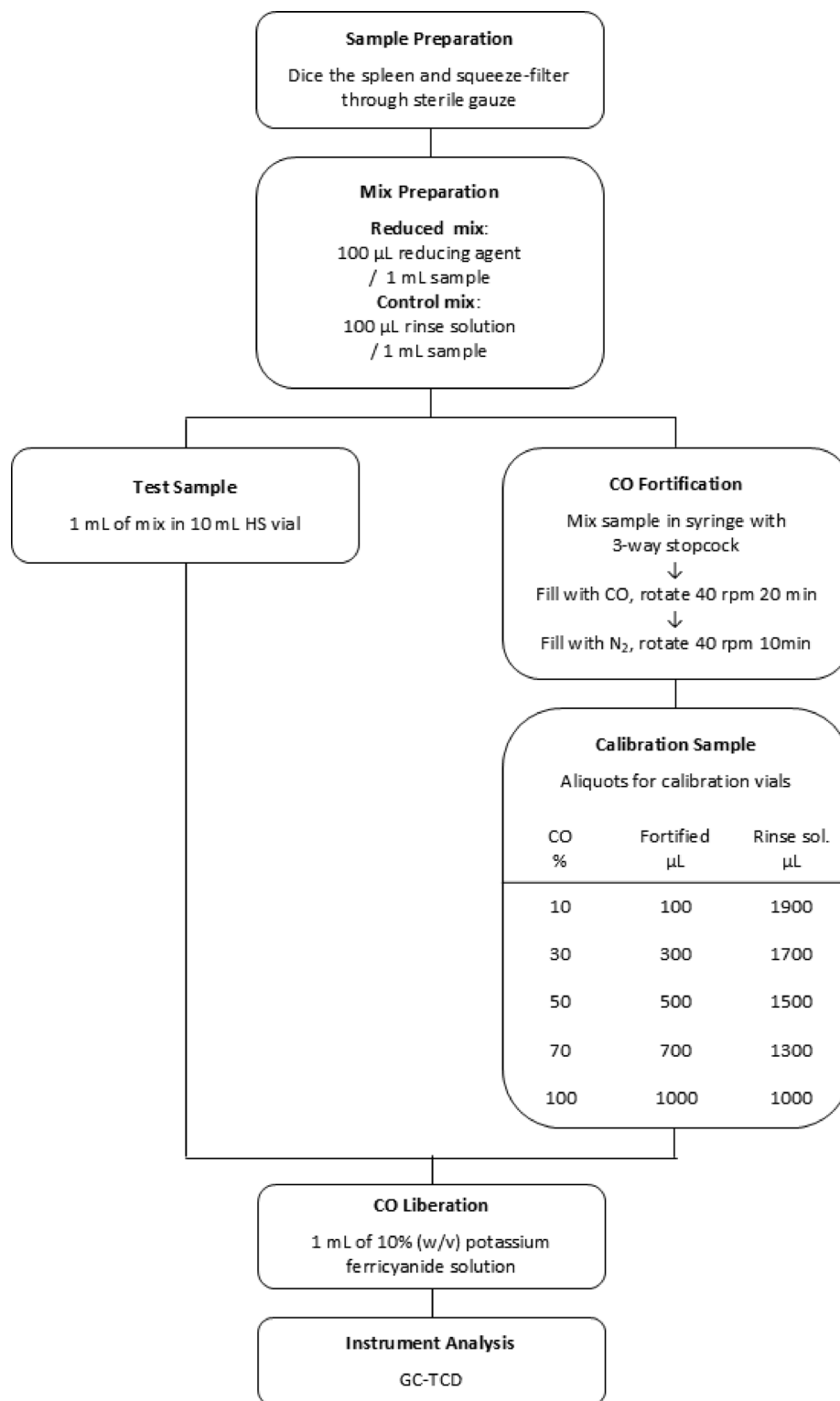


Fig. 1. Flow chart of the sample preparation method.

the reduced sample was 44.2% with a median value of 39.0%. 13 cases had the CO concentration of the control sample between 30% and 50%. The average difference between the control and the reduced sample was 15.5%, with a median value of 14.6%. 20 cases had the CO concentration lower than 30% in the control sample, and the difference between the control and the reduced sample was 7.0% with a median value of 4.8%. Out of all shifted samples, 20 cases had a shift bigger than 20%, and 15 of them had the result of the control sample bigger than 50%.

Parameter	Specification
Instrument	GC-TCD (6890 N, Agilent Technologies, USA)
Instrument Software	GC ChemStation (Rev. B.04.03 16, Agilent Technologies, USA)
Autosampler	Multi-Purpose Autosampler for GC (GERSTEL, Germany)
Column	RT-Msieve 5 A (30 m length × 0.53 mm ID, 50 µm film thickness, RESTEK, USA)
Carrier Gas	Helium (99.999%)
Sample Incubation	60°C, 10 min
Injection	300 µL (gas phase), 200°C, 11 mL/min
Oven	90°C, 16 min
Column Flow Rate	10 mL/min
Detector Temperature	250°C, 25 mL/min

Table 2. GC-TCD analytical Conditions.

Sample	LOD (%)	LOQ (%)	RSD (%)	R ²	Recovery (%)
Spleen A	7.84	12.95	2.66	0.9992	94.91
Spleen B	6.29	8.29	1.86	0.9991	96.73
Blood	4.2	5.27	1.77	0.9959	102.7

Table 3. Validation parameters for the detection of CO using GC-TCD.

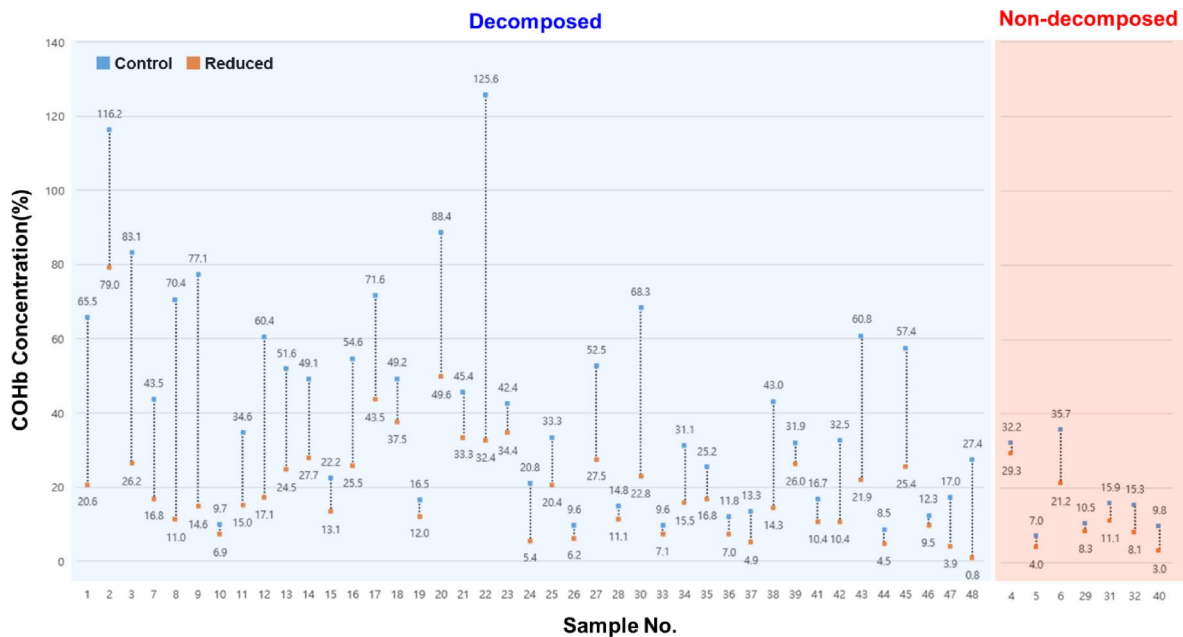


Fig. 2. Result comparison of decomposed and non-decomposed samples.

The 48 samples with shifted results were compared by the samples’ putrefaction. 41 cases labeled as putrefied had a record of putrefaction in the police or the autopsy report, and the other 7 cases labeled as non-putrefied did not. The graph shows the margin between the control and the reduced sample for each label. Those with a margin higher than 40% were all putrefied cases(Fig. 2).

Considering that the CO-positive determination is done when the concentration is higher than 30%¹¹, 19 out of 41 cases in those labeled as putrefied and 2 out of 7 cases in those labeled as non-putrefied had the result changed from CO-positive to CO-negative. In total, 21 cases were diagnosed positive on samples with rinse solutions but negative on the same but reduced sample(Fig. 3). This indicates that in the cases where the sample is heavily decomposed, a false positive diagnosis is possible, and a reducing agent must be incorporated to ensure accurate determination of CO as a cause of death.

The results were put in a table based on the difference value between the control and the reduced sample. The table consists of sample number, gender, age, result of control sample, result of reduced sample, difference

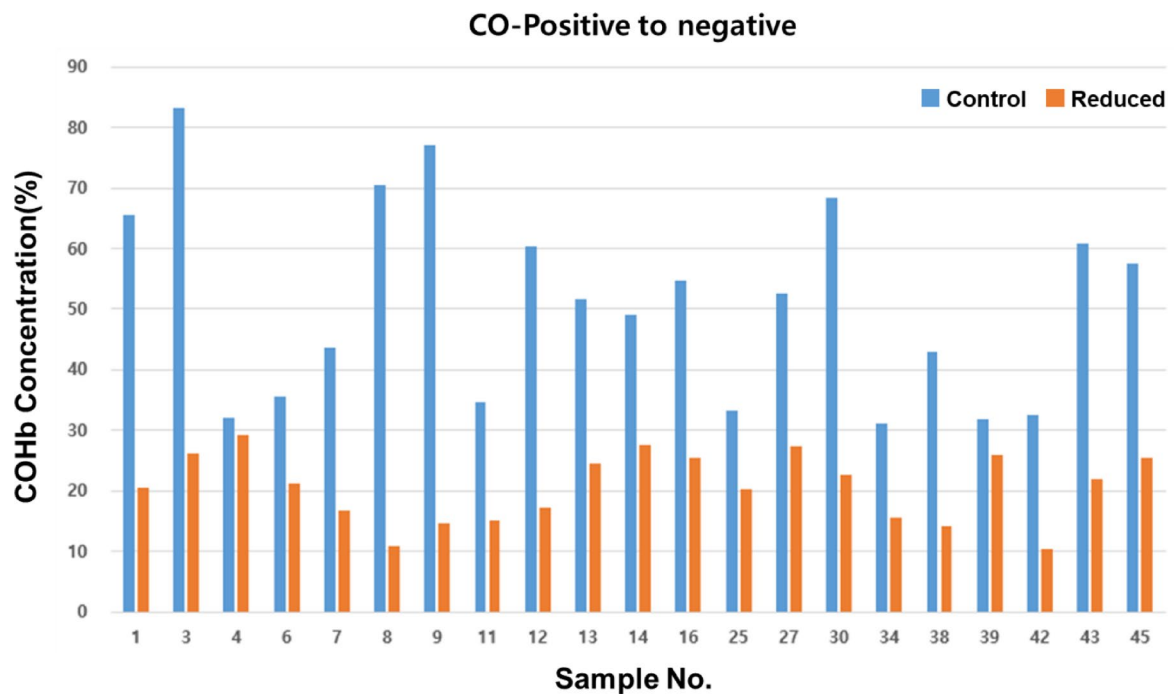


Fig. 3. Result shift from CO-positive to negative.

between the control and reduced sample, decomposition level, and the dates of discovery, autopsy and analysis. When compared by the margin (the result of the control sample – the result of the reduced sample), 20 cases had a margin of less than 10%, 17 cases had a margin between 10 and 30%, and 11 cases had a margin larger than 30% (Table 4(a-c)).

Two extraordinary cases were detected to contain CO higher than 100%, which is theoretically impossible. The results of these cases were lowered when treated with a reducing agent, 125.6–32.4% and 116.2–79.0%. This shows that false results showing over 100% can be prevented by sample reduction.

Statistical analysis

A paired t-test comparing CO concentrations in control and sodium dithionite-treated spleen samples ($n=60$) revealed a statistically significant decrease following reduction (mean control = 33.99%, mean reduced = 17.12%, $p < 0.00000001$). The mean difference in CO concentration between control and reduced spleen samples was 16.87% (SD = 19.39%), resulting in a Cohen's d of 0.87, indicating a large effect size. These results strongly support that MetHb formation leads to overestimation of CO levels, and sodium dithionite reduction produces a substantial and consistent decrease in measured CO levels, likely by reversing methemoglobin-related overestimation.

Discussion

A preliminary test done on blood determined the amount of reducing agent to add. Sodium dithionite was selected as a reducing agent based on previous studies^{14,15,19}. Various concentrations of the sodium dithionite solution were tested on blood samples with high methemoglobin, or normal blood oxidized with sodium nitrite to artificially raise methemoglobin²⁷. The changes in methemoglobin level depending on the concentration of the reducing agent added were analyzed using the oximeter. The optimum amount was 100 μ L of 0.574 M sodium dithionite solution per 1 mL of blood. The same samples were analyzed with GC to confirm that the reduction did not affect the CO concentration of the sample, where the results were stable in both GC and oximeter.

A similar verification test could not be done on spleens for several reasons. First, most spleens can not be analyzed using an oximeter because of the instrument's contamination hazard. In addition, the spleens do not contain enough liquid to be analyzed multiple times. Moreover, CO analysis using GC requires a minimum of 6 to 10 mL, depending on the state of the sample. Thus, in order to test multiple concentrations, at least triple the amount is required, but it is impossible to extract that from average spleen samples. Even if the samples did contain enough liquid, they were less decomposed, meaning that the difference depending on the use of the reducing agent would not show. Therefore, the concentration found in the blood test was used on spleens.

The shifted cases had changes in results for mainly two reasons. One group had similar areas in test samples but a higher area for calibrations, which consisted of 40 cases. The other group had similar areas in calibration samples but lower areas for test samples, which consisted of 8 cases. The changes in the results were the most prominent in 9 cases, which were all part of the first group. There were drastic differences in the calibration curve between reduced and non-reduced samples. The area of the calibration samples' chromatogram had increased

Sample	Gender	Age	Control	Reduced	Difference	Decomposition	Discovery	Autopsy	Analysis
(a) Margin $\leq 10\%$									
4	M	61	32.2	29.3	2.9	None	23.10.23	23.10.25	24.06.02
5	M	59	7.0	4.0	3.0	None	23.10.26	23.10.27	24.06.01
10	M	42	9.7	6.9	2.8	Medium	23.12.21	23.12.22	24.06.01
15	M	32	22.2	13.1	9.1	Medium	24.01.12	24.01.15	24.06.05
19	M	63	16.5	12.0	4.4	Medium	24.02.22	24.02.23	24.06.20
23	F	49	42.4	34.4	8.0	Severe	24.03.08	24.03.11	24.06.07
26	M	47	9.6	6.2	3.4	Medium	24.03.15	24.03.18	24.06.23
28	M	56	14.8	11.1	3.7	Medium	24.03.21	24.03.22	24.06.21
29	M	66	10.5	8.3	2.2	None	24.03.23	24.03.25	24.06.17
31	M	69	15.9	11.1	4.8	None	24.04.07	24.04.09	24.06.20
32	M	29	15.3	8.1	7.1	None	24.04.08	24.04.09	24.06.10
33	M	69	9.6	7.1	2.5	Medium	24.04.11	24.04.11	24.06.19
35	M	59	25.2	16.8	8.4	Medium	24.04.18	24.04.19	24.06.20
36	M	54	11.8	7.0	4.8	Medium	24.04.23	24.04.24	24.06.18
37	M	52	13.3	4.9	8.4	Medium	24.04.23	24.04.25	24.06.19
39	M	51	31.9	26.0	5.9	Medium	24.04.27	24.04.29	24.06.20
40	M	39	9.8	3.0	6.8	None	24.04.28	24.04.29	24.06.18
41	M	59	16.7	10.4	6.3	Medium	24.06.05	24.06.07	24.07.22
44	M	35	8.5	4.5	4.0	Medium	24.06.18	24.06.19	24.07.20
46	M	50	12.3	9.5	2.9	Medium	24.07.05	24.07.08	24.07.25
(b) $10\% < \text{Margin} \leq 30\%$									
6	M	85	35.7	21.2	14.6	None	23.10.30	23.10.31	24.06.04
7	M	36	43.5	16.8	26.8	Medium	23.10.31	23.11.01	24.06.04
11	M	63	34.6	15.0	19.6	Medium	23.12.28	23.12.29	24.06.01
13	M	58	51.6	24.5	27.1	Medium	24.01.07	24.01.09	24.06.03
14	M	47	49.1	27.7	21.4	Medium	24.01.08	24.01.09	24.06.23
16	M	32	54.6	25.5	29.1	Medium	24.01.30	24.02.01	24.06.21
17	F	59	71.6	43.5	28.1	Medium	24.02.09	24.02.12	24.06.21
18	M	60	49.2	37.5	11.7	Medium	24.02.19	24.02.20	24.06.07
21	M	44	45.4	33.3	12.1	Medium	24.03.05	24.03.07	24.06.22
24	M	68	20.8	5.4	15.4	Medium	24.03.11	24.03.12	24.06.18
25	M	53	33.3	20.4	12.9	Medium	24.03.14	24.03.15	24.06.20
27	M	56	52.5	27.5	25.1	Medium	24.03.19	24.03.21	24.06.04
34	M	47	31.1	15.5	15.6	Medium	24.04.16	24.04.17	24.06.20
38	M	27	43.0	14.3	28.7	Medium	24.04.24	24.04.26	24.06.23
42	M	51	32.5	10.4	22.1	Medium	24.06.07	24.06.10	24.07.21
47	M	57	17.0	3.9	13.1	Severe	24.08.27	24.08.29	24.09.20
48	F	55	27.4	0.8	26.5	Severe	24.09.13	24.09.18	24.09.20
(c) $30\% < \text{Margin}$									
1	M	65	65.5	20.6	44.9	Medium	23.01.08	23.01.10	24.06.04
2	F	38	116.2	79.0	37.2	Medium	23.02.02	23.02.06	24.06.10
3	M	52	83.1	26.2	56.9	Medium	23.05.07	23.05.08	24.06.10
8	M	32	70.4	11.0	59.4	Medium	23.12.07	23.12.08	24.05.31
9	M	49	77.1	14.6	62.6	Medium	23.12.07	23.12.08	24.05.31
12	M	36	60.4	17.1	43.3	Severe	23.12.28	24.12.29	24.06.04
20	M	56	88.4	49.6	38.8	Severe	24.02.27	24.02.28	24.06.22
22	M	53	125.6	32.4	93.2	Medium	24.03.08	24.03.11	24.06.03
30	M	64	68.3	22.8	45.5	Severe	24.04.01	24.04.02	24.06.22
43	M	59	60.8	21.9	39.0	Severe	24.06.09	24.06.10	24.07.25
45	M	54	57.4	25.4	32.0	Medium	24.07.04	24.07.05	24.07.25

Table 4. CO results and the specifics of the sample grouped by the margin.

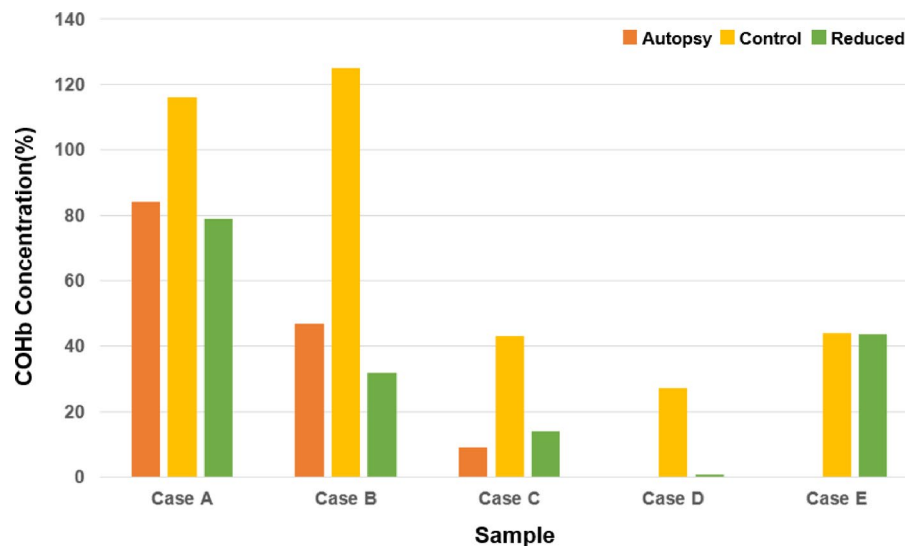


Fig. 4. Case study of result at the time of autopsy, control and reduced sample.

	Autopsy	Control	Reduced	CO relevance	Decomposition
Case A	84	116	79	Charcoal	Medium
Case B	47	125	32	Charcoal	Medium
Case C	9	43	14		Medium
Case D		27.4	0.8		Severe
Case E		43.9	43.6	Charcoal	None

Table 5. Comparison of CO results upon autopsy and reducing agent experiment.

in the reduced sample, and the changes were more evident in higher calibration points, leading to a more linear calibration curve. This can be easily noticed by comparing the area of the 100% calibration sample.

Sample number 8 had a calibration point area of 100% 86.8 with a rinse solution and 654.8 with a reducing agent. The linearity of the calibration curve increased about eight times from 0.877 to 6.655. The result changed from 70.45% with rinse solution to 11.01% with reducing agent, even though the test sample area was similar to 61.8 with rinse solution and 67 with reducing agent. Through this, it can be assumed that this sample was high in methemoglobin, and the reduction affected the fortification and the result.

A similar pattern has been observed in the other 8 cases where the area of 100% calibration point increased and was 2.1 to 4.6 times larger in the reduced sample, resulting in the calculated CO being lowered.

For the other group with a difference in the area of the test samples, the changes in the calibration curve were less evident. The calculated results differed because the area of the test samples was lower in the reduced samples.

The five cases requested for CO analysis at the time of autopsy were used to compare the results from then and when the reducing agent was tested (Fig. 4).

Case A was a 38-year-old female found putrefied in a tent with charcoal briquettes, which indicates CO intoxication. Tested at the time of autopsy, CO was 84%, and 12 months later, the non-reduced control sample with rinse solution resulted in CO of 116%. However, when treated with the reducing agent, the result was 79%, which is similar to the original result. Case B was a 53-year-old male found putrefied at home with charcoal briquettes. Tested at the time of autopsy, CO was 47%, and three months later, the non-reduced control sample had CO of 125%. However, when treated with a reducing agent, the result was 32%, which is significantly lower than the original result but still determined as CO-positive. Case C was a 27-year-old male found putrefied at home with no evidence of CO poisoning. When tested at the time of autopsy, CO was 9%, but when stored for 3 months, it rose to 43%. However, when treated with a reducing agent, it went down to 14%. The analysis of these cases proves that the sample must be analyzed as fast as possible, and storage may produce methemoglobin, which affects the result and determines a false-positive even if the container is tightly sealed and refrigerated at 4°C²⁶. Case D was a 55-year-old female found heavily putrefied at home. When tested at the time of autopsy, CO was 27%, but when treated with a reducing agent, the result was as low as 0.83%, showing practically no CO. Case E was a 62-year-old male found with charcoal briquettes at home (Table 5).

Tested at the time of autopsy, CO was 43.9%, and when treated with a reducing agent, the result was similar, 43.6%. The little difference is assumed to have been from the spleen sample not being decomposed, unlike other cases, where not much MetHb has formed to affect the fortification process. The results of the two cases show the relationship between putrefaction and MetHb by comparing the changes in results depending on the use of the reducing agent. The sample with severe putrefaction has high MetHb, and the result is heavily affected by the use

of a reducing agent. In contrast, the sample with no putrefaction does not contain much MetHb, and, therefore, a reducing agent does not have any effect on the result.

Limitations

While this study demonstrates a clear and reproducible reduction in CO overestimation through sodium dithionite treatment, certain limitations should be noted. Direct quantification of methemoglobin (MetHb) in spleen samples was not performed due to instrumental limitations. While an oximeter capable of measuring MetHb levels is available, its analysis depends on the optical clarity and homogeneity of the sample, conditions typically met in fresh liquid blood but not in postmortem spleen tissue, especially in decomposed cases where the consistency is often semi-solid. Additionally, the GC-TCD setup used in this study is not equipped to distinguish specific hemoglobin species. Therefore, the MetHb interference was assessed indirectly by comparing CO concentrations in treated and untreated samples from the same tissue source.

Although several postmortem factors can interfere with CO quantification, such as thermocoagulation, putrefaction, and postmortem CO production, specific measures were taken to minimize their influence. None of the included cases involved direct fire exposure, and even those associated with combustion resulted in CO intoxication rather than thermal injury. Our rinse solution contained a mild hemolytic agent, which minimized protein coagulation under the analytical conditions used. To reduce the risk of misinterpretation due to postmortem CO formation, samples were classified as CO-positive when CO content exceeded 30%. Additionally, all spleens were analyzed in parallel (control vs. reduced), ensuring that any decomposition-related CO would affect both aliquots equally, without biasing the comparative results.

Conclusion

The carbon monoxide analysis of tissue using GC-TCD often yields incorrect results when the sample is severely putrefied because of the increased methemoglobin level. This study was conducted to test the effect of sample reduction for mitigating the impact of high methemoglobin levels by treating the sample with a sodium dithionite solution, a reducing agent, and comparing it with the result of the control sample. The findings showed that incorporating a reducing agent considerably lowered the result and prevented false-positive diagnoses on putrefied samples. Therefore, the CO analysis from a putrefied tissue should include a reduction process in the sample preparation procedure to lower the methemoglobin level and ensure CO fortification. Moreover, in order to ensure accurate analysis, cross-checking is recommended by analyzing the duplicate sample with and without a reduction process in GC analysis of CO concentration in a tissue sample.

Data availability

All the data are available on request from the corresponding author.

Received: 8 February 2025; Accepted: 15 July 2025

Published online: 22 July 2025

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Acknowledgements

This work was supported by National Forensic Service (NFS2024CHE01), Ministry of the Interior and Safety, Republic of Korea.

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Conceptualization: M.L., Y.J.; Data curation: M.L., D.L.; Methodology: M.L., D.L.; Resources: M.L., Y.J.; Writing-original draft: M.L., D.L.; Validation: H.J.K.; Project administration: W.P. All authors have reviewed and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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